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TITLE: Novel Paclitaxel Copolymer for Treatment of Androgen-
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INTRODUCTION

Androgen-independent (AI)/hormone refractory metastatic disease is a virtually inevitable endpoint for prostate cancer (PCa), frequently associated with p53 and androgen-receptor (AR) mutations, Bcl-2 over-expression, and possibly other genetic and epigenetic alterations that confer treatment resistance. Despite the enormity of this clinical issue, regrettably, effective therapeutic options are few. Numerous clinical trials have documented activity for certain drug regimens, including those affecting the microtubular apparatus, such as the taxanes (docetaxel/taxotere and paclitaxel), estramustine phosphate and vinblastine, typically in combination with a platinum agent and/or etoposide. It is to be hoped that this clinical activity and true survival benefit with existing drugs will be verified in ongoing phase III trials. Nevertheless, none of these trials have evaluated passively or actively tumor-targeted paclitaxel prodrugs in AI PCa.

Drug copolymers are high molecular weight conjugates that can be actively transported to the endosome, where they are then cleaved/activated to release free drug at this organelle. For DNA-targeting drugs, this may afford superior nuclear access compared to import via diffusion as occurs with free drug. Further, it restricts the gradient of export of conjugate-released drug via membrane-localized drug efflux mechanisms, e.g., P-gp170, that are clearly operant on free drug. This approach has proven to be successful for doxorubicin *in vitro*.

In vivo, other considerations may be more relevant, including distribution to tumor vs. normal tissue. High molecular weight drug copolymers may, on the one hand, 1) restrict diffusion-controlled uptake by normal tissues that occurs with free drug; but, on the other, they may 2) allow efficient extravasation across the abnormal tumor endothelium, thereby enhancing tumor localization compared to free drug. This has been substantiated for poly(L-glutamic acid)-paclitaxel (PGA-TXL) in several pre-clinical studies.

In a broader context than originally presented in this proposal, we have recently become aware of and begun to develop evidence for the so-called "sphingolipid rheostat" model. In this model, the balance between pro-apoptotic sphingolipids, ceramide and sphingosine, and the anti-apoptotic or pro-survival sphingolipid, sphingosine-1-phosphate (S-1-P), determines cell fate.

A key modulator of this rheostat is sphingosine kinase (SpK), which phosphorylates sphingosine to S-1-P; *in vivo*, over-expression of SpK has been shown to promote tumorigenesis (1). Higher microvessel density was observed peripherally in the SpK-transfected tumors. SpK has been reported to mediate VEGF-mediated activation of Ras and MAPK activity in T24 bladder cells (2). In this model, VEGF activated SpK, reduced Sp while raising S-1-P levels, and caused accumulation of Ras-GTP and phospho-ERK1/2; siRNA targeted to SpK blocked these responses. Raf kinase inhibition, SpK inhibition, dominant-negative SpK and siRNA directed to SpK all blocked VEGF induction of phospho-ERK1/2. Thus, SpK could couple signaling through PKC to Ras in these cells (2). Overexpression of SpK in a Matrigel system of angiogenesis resulted in vascular maturation and angiogenesis (3).

Thus, this SpK-regulated pathway and the sphingolipid rheostat model are of interest due to possible interactions with chemotherapeutic agents (e.g., Taxol) in apoptotic signaling, as well as in regulation of tumor angiogenesis. **For the latter reason, we have slightly delayed undertaking Task 1, involving characterization of angiogenesis in MDA-PCa models, until we have a somewhat better understanding of the SpK-regulated pathway *in vitro*.** The sphingolipid, dimethyl-sphingosine (DMSP), is the most potent known lipid inhibitor of SpK; DMSP is also apoptogenic by itself, and we have initially focused on characterization of this pathway, as it should provide insight to optimally combining it with paclitaxel-mediated therapy.

S-1-P, the “mediator” of SpK over-expression, has been shown to inhibit paclitaxel-induced caspase-3 activation in other tumor models. We have begun to evaluate the possible role of this rheostat in determining the apoptotic sensitivity of human prostate carcinoma cell lines to paclitaxel *in vitro*. This may have *in vivo* relevance, as well, since we should be able to determine the effects of combination paclitaxel/DMSP regimens in human prostatic carcinoma xenograft models.

In these studies, two paclitaxel copolymers will be evaluated. The first copolymer to be employed, PGA-TXL, has shown both reduced toxicity and greater tumor localization compared to Taxol in several animal models, thereby fulfilling two expectations of copolymer behavior. PGA-TXL should be considered non-targeted, as there is no known receptor for its uptake, and most like is endocytosed by pinocytosis. The other paclitaxel copolymer, hyaluronic acid-paclitaxel (HA-TXL), is based on an HA backbone that will also serve as a ligand for receptor-mediated uptake by CD44. However, it should also be endocytosed via fluid-phase pinocytosis. In this proposal, we will establish the toxicity, pharmacokinetics and anti-tumor efficacy of these paclitaxel copolymers in human prostatic adenocarcinoma xenograft models in nude mice; we will also consider combination treatment with liposomal-DMSP.

BODY

Since the original submission of this grant, we have become increasingly interested in hyaluronic acid-paclitaxel (HA-TXL). As a paclitaxel copolymer; compared to PGA-TXL, it has the additional potential to be incorporated by CD44(+) cells via receptor-mediated uptake, aside from uptake by pinocytosis. Subsequent to becoming aware of the prior activity and reports in this field (4, 5), we have tried to develop novel HA-TXL constructs using alternative linker strategies and other techniques. We also attempted to couple to a different backbone, chondroitin/chondroitin sulfate, also ligands for CD44. The intent was to develop a strong intellectual property position, as our long-term goal is also drug development; a patent position would greatly facilitate this endeavor.

After more than a year at these efforts, we have come to the conclusion that the prior reported chemistry for the synthesis of HA-TXL is indeed quite appropriate and comprehensive, as we have so far been unsuccessful in supplanting it. Further, for reasons we cannot yet ascertain, the attempts to couple paclitaxel or paclitaxel adducts to chondroitin/chondroitin sulfate simply do not proceed. We do not believe that it is due to the presence of the sulfate group.

Hence, we have now returned to coupling HA to the N-hydroxy-succinimide ester of paclitaxel via a hydrazide linkage, following the procedure of Prestwich and coworkers (4, 5). These efforts have already met with success in our pilot syntheses, allowing us to conduct initial *in vitro* studies. In fact, Dr. Prestwich's group has engaged in collaboration with a group at George Washington University; they presented a poster at the 2003 AACR meeting on this subject. Their study did NOT deal with any human prostatic carcinoma xenograft models, however, so our main work is in NO way pre-empted.

In these studies, we first evaluated the responses of the human PC-3 prostatic carcinoma cell line, which is capable of growth as a xenograft model, to a concentration range of Taxol and PGA-TXL, with assessment of HA-TXL to follow. We anticipated that the response to the prodrug would be more protracted than the response to Taxol, due to more immediate availability of free paclitaxel with the latter.

We observed that the losses in survival of PC-3 cells (as determined by dye assays) were time- and paclitaxel concentration-dependent, with generally low negative slopes on the concentration curves (Fig. 1). The IC_{50} for both Taxol and PGA-TXL was ~ 500 ng/ml (paclitaxel equivalents); lower concentrations of PGA-TXL were still capable of causing losses of survival. A similarity in IC_{50} values, also observed for NMP-1 and HEY human ovarian tumor cell lines, did NOT parallel the sensitivity of NMP-1 tumors to these drugs *in vivo* (6); PGA-TXL was active against NMP-1 and HEY tumor models, whereas they were highly Taxol-resistant (6). This underscores the favorable tissue pharmacokinetics of PGA-TXL in this setting.

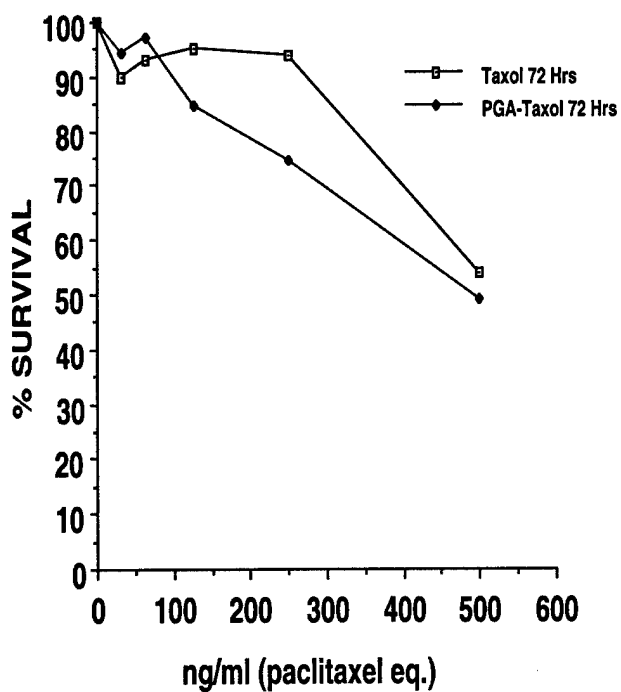


Figure 1

We have also evaluated the responses of the MDA-PCa 2a and MDA-PCa 2b cell lines to HA-TXL (Fig. 2). These lines demonstrated similar sensitivities to HA-TXL ($IC_{50} < 400$ ng/ml), and slightly greater sensitivity than observed above with PC-3 cells and Taxol and PGA-TXL.

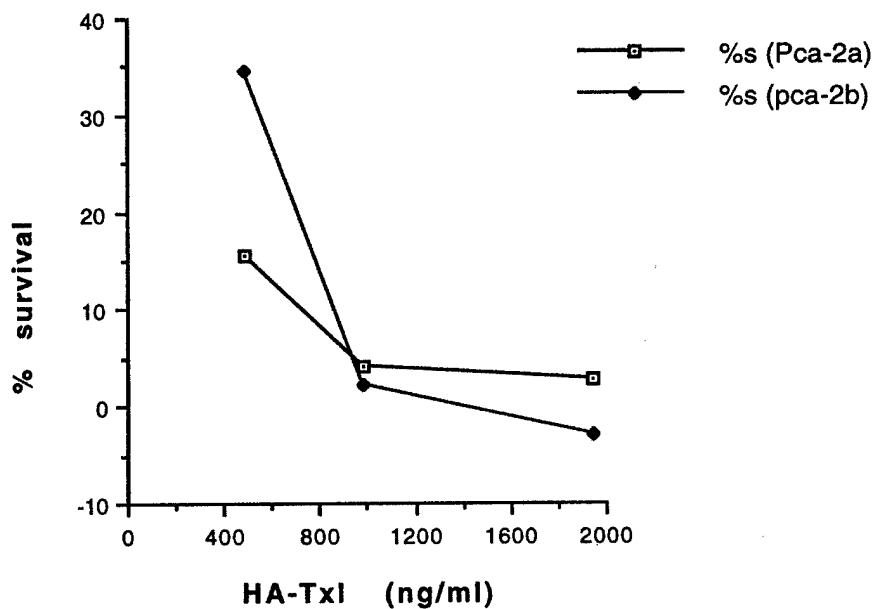


Figure 2

With these *in vitro* responses now characterized, following appropriate paclitaxel copolymer scale-up, we will be poised to undertake **Task 2, involving pharmacokinetic analyses of drug accumulation in PC-3 and MDA-PCa bone models.**

The so-called “sphingolipid rheostat” model involves pro-apoptotic, ceramide and sphingosine, and anti-apoptotic or pro-survival, S-1-P (7-9). S-1-P has been shown to inhibit caspase-3 activation by paclitaxel, a significant reason for our interest in merging this area with the current Aims. SpK, which phosphorylates sphingosine to S-1-P, is potently inhibited by DMSP; DMSP is also apoptogenic by itself. We have begun to evaluate the possible role of this rheostat in determining the apoptotic sensitivity of human prostatic carcinoma cell lines to paclitaxel *in vitro*. This may have *in vivo* relevance, as well; since we have been able to induce anti-tumor effects using liposomal-DMSP as monotherapy in a human breast adenocarcinoma orthotopic xenograft model (10), we should be able to evaluate the effects of combination targeted paclitaxel/DMSP regimens in our human prostatic carcinoma xenograft models, enhancing the novelty and efficacy of our approach.

The first response of PC-3 cells that we evaluated relevant to these efforts has particular relevance to prostate cancer: BCL-2-mediated therapeutic resistance. In Figure 3 is shown the survival response following exposure to sphingosine, of parental PC-3 cells, as well as a series of cell lines derived following stable transfection with *bcl-2*. The results indicate, surprisingly, that BCL-2 has no detectable protective effects against this pro-apoptotic mediator.

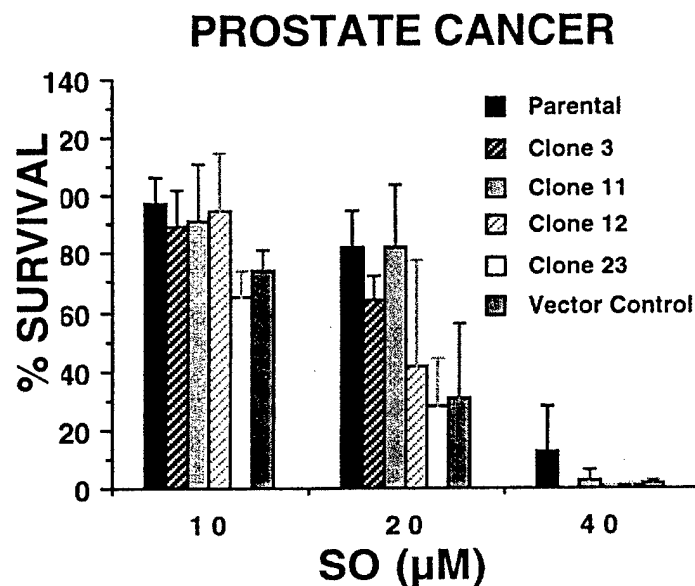


Figure 3

The next mechanistic study evaluated early steps in the response of parental PC-3 cells to DMSP. PC-3 cells were incubated with DMSP for 4 hr prior to assaying for caspase activation using the CaspaTag flow cytometric assay. Dose-dependent activation of caspases by DMSP was observed (Fig. 4).

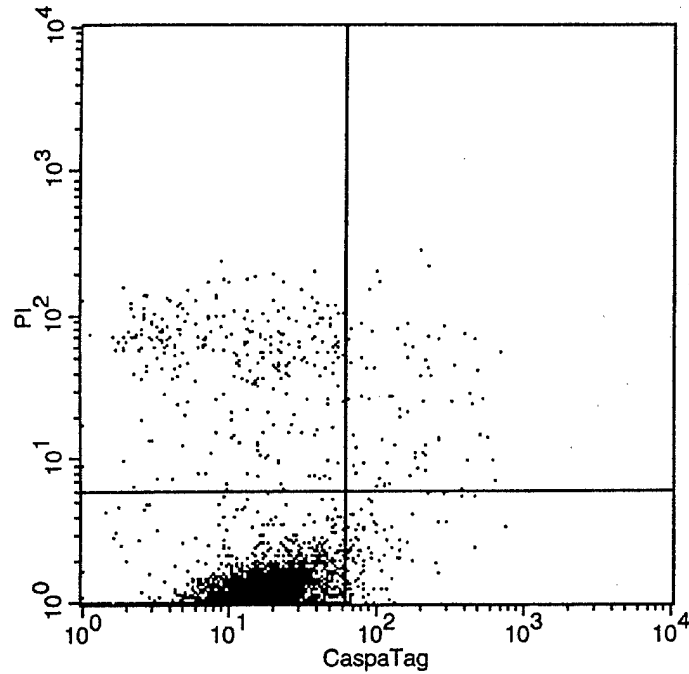


Figure 4, Panel A Control cells; 2.9%, 0.7% and 1.0% cells in the upper left (UL), upper right (UR) and lower right (LR) quadrants, respectively.

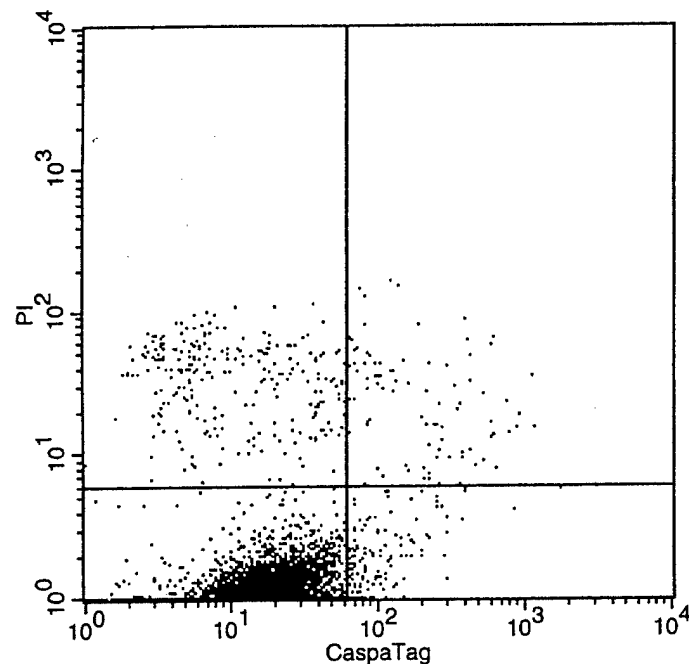


Figure 4, Panel B Cells treated with 10 uM DMSP; 2.6%, 0.9% and 1.4% cells in the UL, UR and LR quadrants, respectively.

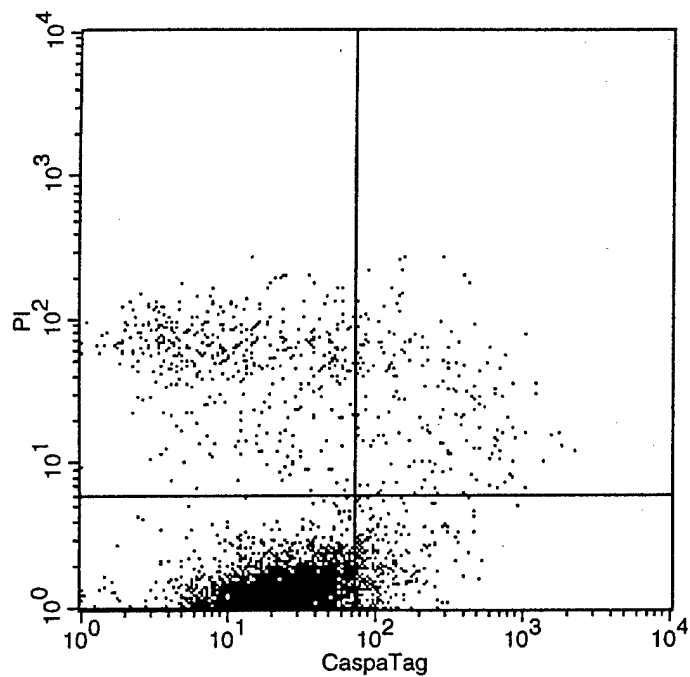


Figure 4, Panel C Cells treated with 20 uM DMSP; 4.6%, 1.9% and 3.1% cells in the UL, UR and LR quadrants, respectively.

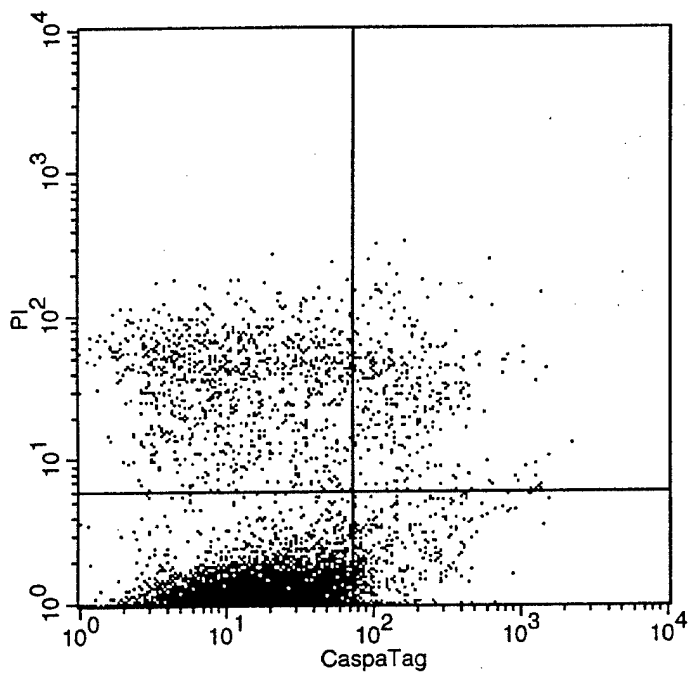


Figure 4, Panel D Cells treated with 30 uM DMSP; 9.6%, 3.4% and 4.2% cells in the UL, UR and LR quadrants, respectively.

Similar dose- and time-dependent activation of caspase-9 in PC-3 cells by DMSP was observed (**Table 1**). This suggested that the mitochondria might be a specific and upstream target of DMSP, triggering apoptosome formation and caspase-9 activation (see Fig. 6).

TABLE 1

ACTIVATION OF CASPASE-9 IN PC-3 CELLS BY DMSP

TREATMENT	UL	UR	LR
CONTROL	6.3 ± 0.3	1.0 ± 0.1	0.5 ± 0.1
20 UM, 0.5 HR	14.9 ± 2.9	7.2 ± 2.5	1.5 ± 0.1
30 UM, 0.5 HR	21.6 ± 4.7	12.8 ± 2.5	2.9 ± 1.0
20 UM, 1.0 HR	19.2 ± 4.9	2.4 ± 0.1	1.7 ± 0.8
30 UM, 1.0 HR	29.8 ± 5.3	12.1 ± 1.2	2.5 ± 0.3
20 UM, 2.0 HR	28.4 ± 4.3	6.5 ± 1.4	2.0 ± 0.3
30 UM, 2.9 HR	45.6 ± 1.0	14.4 ± 0.1	2.7 ± 0.1

Parental PC-3 cells were incubated with 30 μ M DMSP for 6 hr, with and without the broad-spectrum caspase inhibitor, Z-VAD (10 μ M), prior assaying for TUNEL-positive cells using flow cytometric assay (Figure 5).

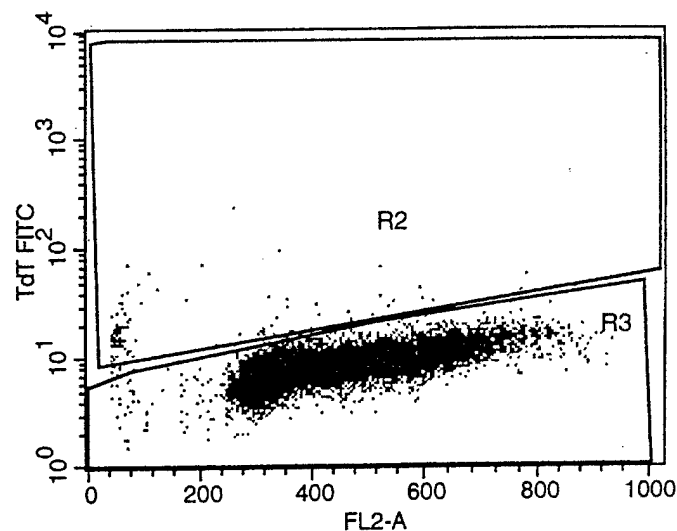


Figure 5, Panel A: Control cells; 0.5% TUNEL-positive (R2 population).

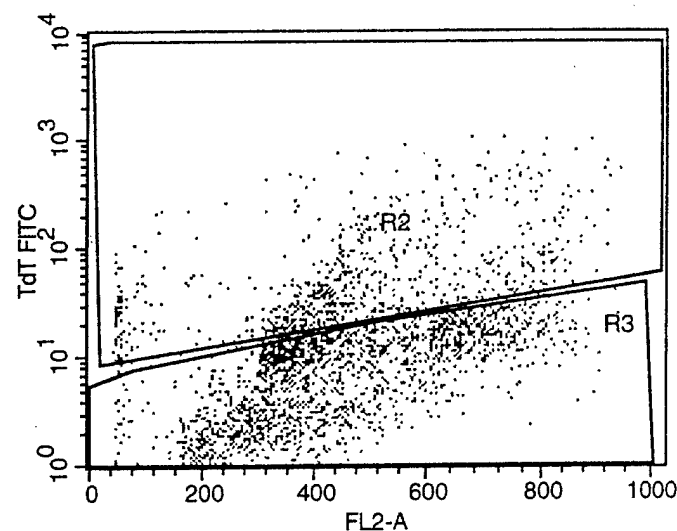


Figure 5, Panel B: Cells treated with 30 μ M DMSP; 28.6% TUNEL-positive.

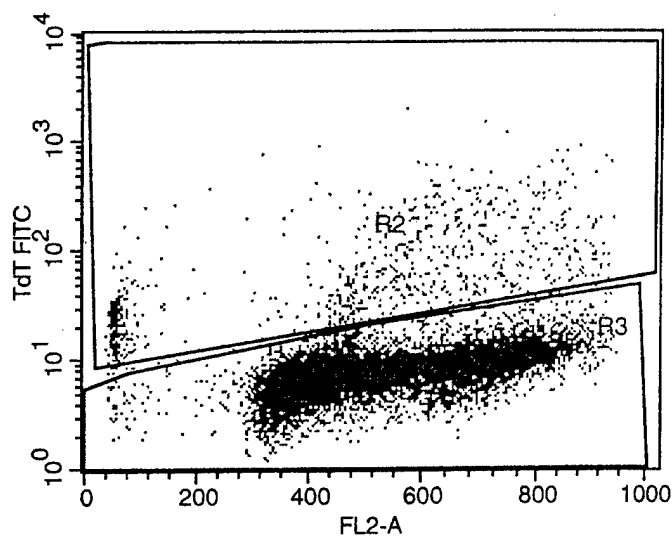


Figure 5, Panel C: Cells treated with 30 μ M DMSP + 10 μ M Z-VAD; 6.1% TUNEL-positive

DMSP induced DNA fragmentation in PC-3 cells, as evidenced by detection of TUNEL-positive cells, and this fragmentation could be significantly blocked with Z-VAD. Production of Annexin-positive cells by DMSP could NOT be blocked with Z-VAD (data not shown). DMSP-induced development of TUNEL-positive and hypodiploid cells was not diminished in BCL-2 over-expressing clones (data not shown).

Parental PC-3 cells and BCL-2 over-expressing PC-3 tranfectants, #3 and #11, were treated with DMSP at various concentrations for 2 hr prior to staining with the mitochondrial-specific dyes, Mitotracker Green and CMX-Ros. Two-color flow cytometric diagrams are shown (Fig. 6).

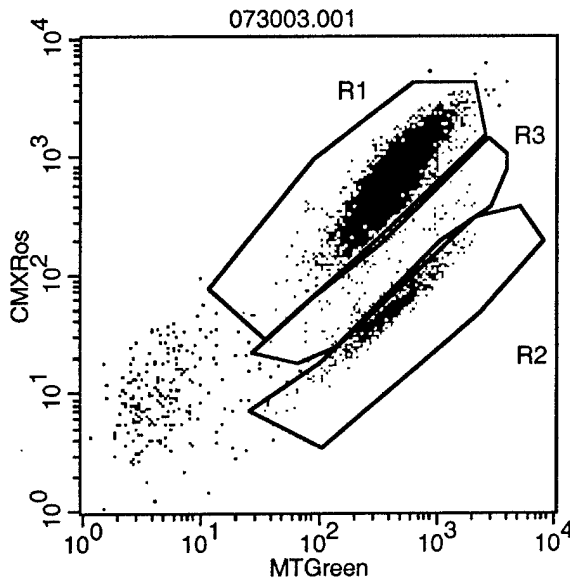


Figure 6, Panel A: Control parental PC-3 cells; 89.2% in R1, 6.4% in R2, and 1.3% in R3

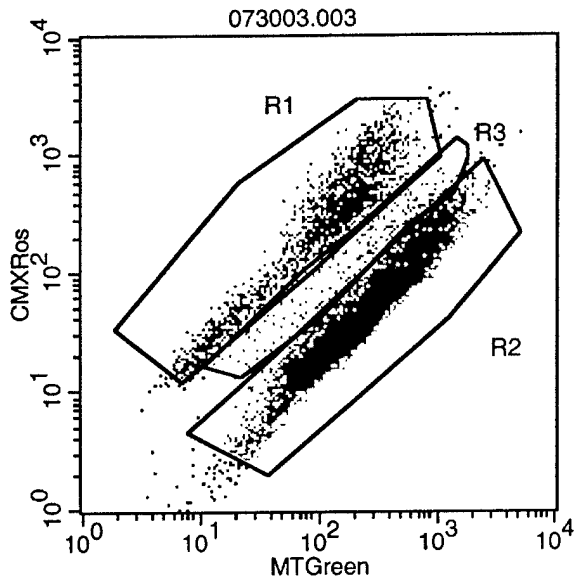


Figure 6, Panel B: Parental PC-3 cells treated with 30 uM DMSP for 2 hr; 19.0% in R1, 77.5% in R2, and 3.0% in R3

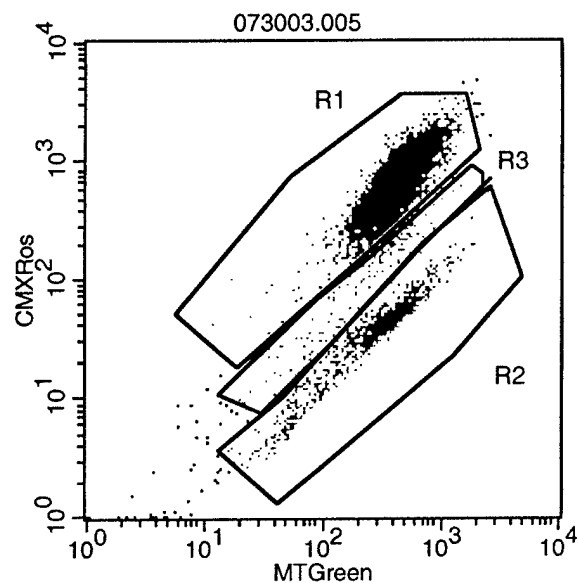


Figure 6, Panel C: Control PC-3 Clone #3 transfectants; 88.1% in R1, 9.1% in R2, and 1.7% in R3

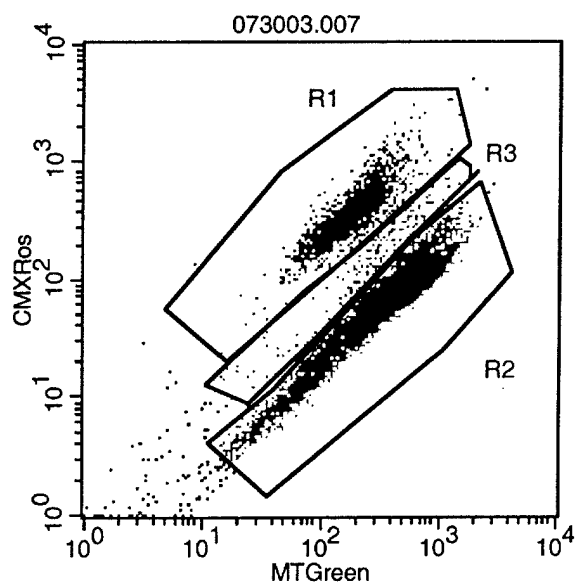


Figure 6, Panel D: PC-3 Clone #3 transfectants treated with 30 uM DMSP for 2 hr; 18.8% in R1, 77.4% in R2, and 2.0% in R3

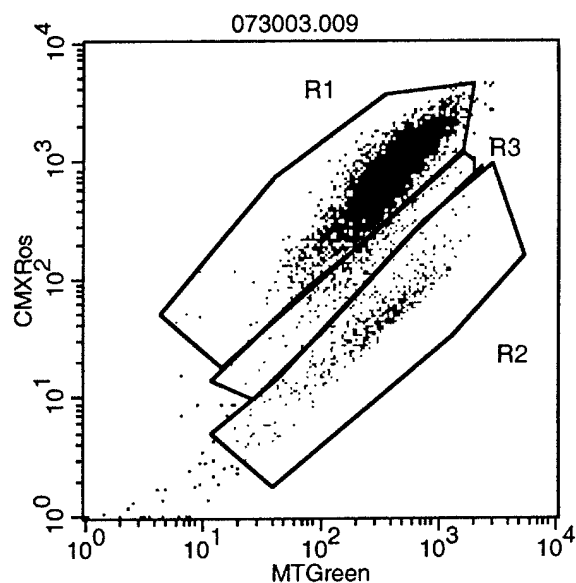


Figure 6, Panel E: Control PC-3 Clone #11 transfectants; 92.3% in R1, 4.6% in R2, and 2.8% in R3

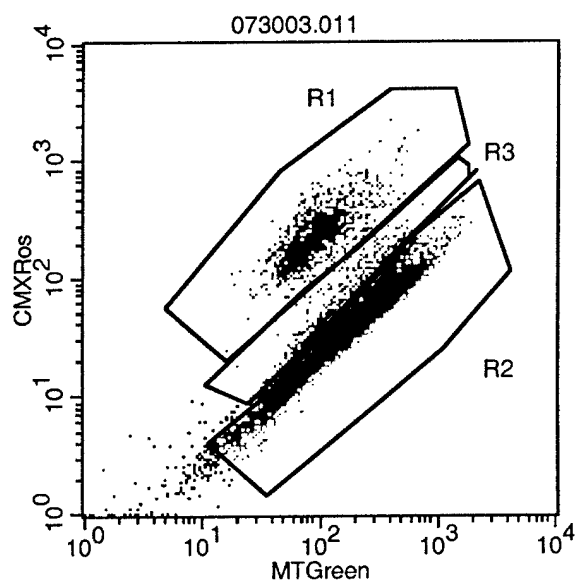


Figure 6, Panel F: PC-3 Clone #11 transfectants treated with 30 uM DMSP for 2 hr; 12.0% in R1, 77.3% in R2, and 4.8% in R3

DMSP induced dose-dependent loss of mitochondrial function in PC-3 cells that was NOT protected by BCL-2 over-expression.

PC-3 cells were treated with selected concentrations of DMSP for 2-18 hr. They were subsequently treated with digitonin to permeabilize the plasma membrane and release cytoplasmic cytochrome C, and then immunostained for residual mitochondrial cytochrome C prior to flow cytometric analyses (11).

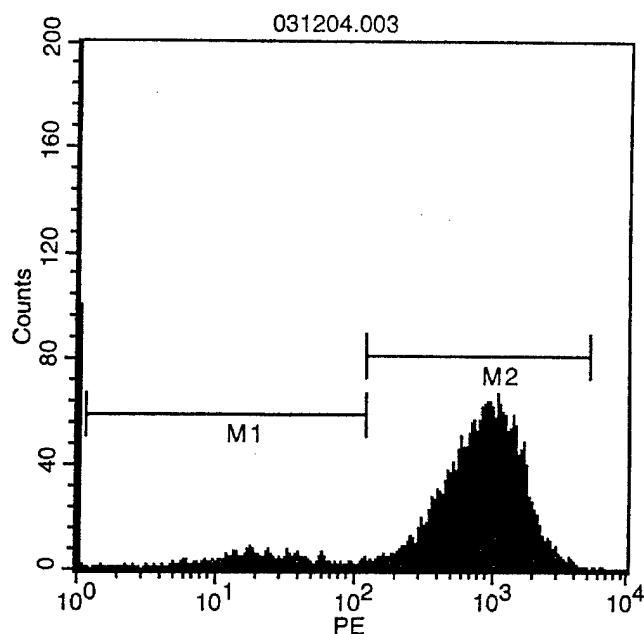


Figure 7, Panel A: Control parental PC-3 cells, treated with digitonin only; 8.2% in M1 and 88.2% in M2.

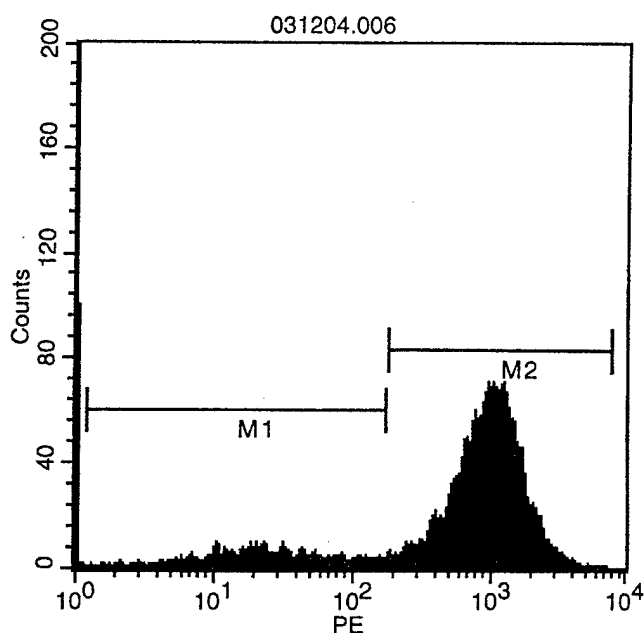


Figure 7, Panel B: PC-3 cells treated for 2 hr with 10 uM DMSP; 14.1% in M1 and 80.8% in M2.

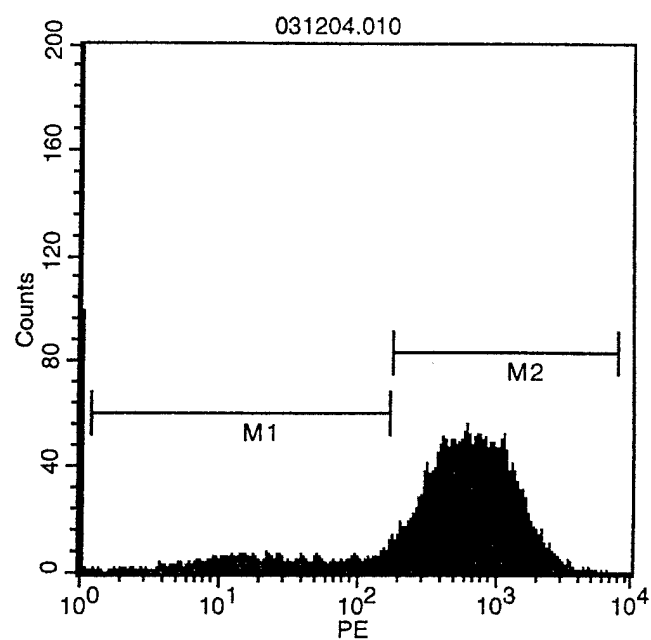


Figure 7, Panel C: Cells treated for 2.0 hr with 20 uM DMSP; 13.5% in M1 and 82.7% in M2.

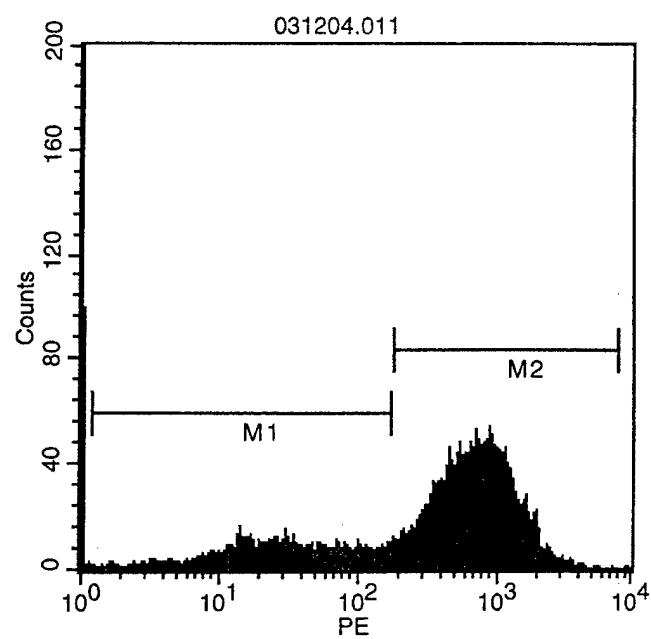


Figure 7, Panel D: Cells treated for 2 hr with 30 uM DMSp; 22.8% in M1 and 71.1% in M2.

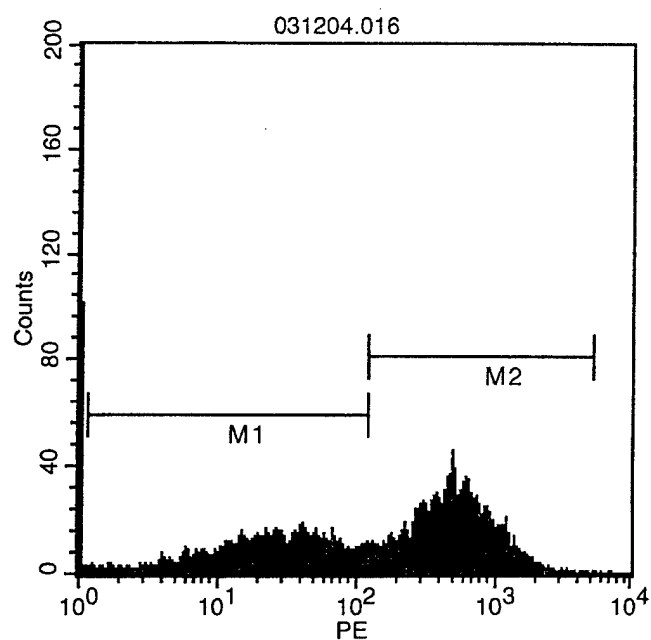


Figure 7, Panel E: Cells treated for 18 hr with 15 μ M DMSp; 32.1% in M1 and 46.7% in M2.

DMSp induced rapid, dose-dependent loss of mitochondrial cytochrome C in PC-3 cells.

In investigations distinct from, but potentially useful for, this proposal, we have obtained data highly relevant to systemic delivery of DMSP to tumor xenografts, in support of the anti-tumor efficacy of DMSP-based monotherapy. MDA-MB-361 human HER-2/neu-over-expressing breast adenocarcinoma cells were implanted orthotopically in the mammary fat pad of female nude mice. Treatment began when tumors had grown to 4-6 mm diameter, at which time the tumor vasculature should have been well-developed, and long-circulating liposomal formulations of DMSP should have been able to exploit the EPR effect. Mice were randomized into control and DMSP treatment groups. DMSP/DPPC/DSPC (1:2:2, mole ratio) SUV formulations, with ~8 mole percent DSPE-PEG added to increase circulation time, were then administered in five weekly i.v. injections (4.5 mg DMSP per injection). Treatment was apparently well-tolerated by the mice as judged by lack of significant clinical symptoms.

We used a Kaplan-Meyer survival analysis, with the day of humane sacrifice due to tumor burden as the event time. Logrank (Mantel-Cox) analysis, using censoring for the surviving treated mice, gave a p value of <0.08 for treatment vs. control groups. The basis for missing significance at 0.05 may likely be due to the small sample size, but perhaps is also suggestive of the existence of two distinct phenotypes in the treatment group: those that were highly refractory and grew through treatment, and those that were apparently cured (or were at least highly responsive). Cumulative and Ln cumulative hazard plots for the control group and the non-responding treated mice were close to parallel (similar slopes), confirming the refractory nature of the latter. Logrank analysis for responders vs. controls gave a p value well below 0.05. Surviving treated mice are still being tracked at > 20 weeks.

The antitumor activity of DMSP in this setting could be attributed to: 1) direct apoptogenic effects on the tumor cells; 2) reduction of the apoptotic threshold in tumor cells due to inhibition of SpK and resultant down-regulation of S-1-P-mediated pro-survival pathways; and/or 3) anti-angiogenic effects of SpK inhibition.

As we consider merging the paclitaxel copolymer strategy with the spingolipid/sphingolipid rheostat strategy, the ability to systemically deliver DMSP to mediate its pro-apoptotic/anti-angiogenic mechanisms becomes quite appealing for treatment of a systemic tumor model such as the MDA-PCa bone models.

KEY RESEARCH ACCOMPLISHMENTS

- Documented the sensitivity of human prostatic carcinoma cell lines, PC-3, MDA-PCa 2a and MDA-PCa 2b to Taxol, PGA-TXL and HA-TXL
- Documented the susceptibility of PC-3 cells to exogenous sphingolipids, and the ability of these sphingolipids to induce BCL-2-independent cell death
- Documented DMSP-induced caspase activation in PC-3 cells; in particular, early activation of caspase-9
- Documented DMSP-induction of TUNEL-positive cells, and the ability of the broad-spectrum caspase inhibitor, Z-VAD, to block this induction in PC-3 cells
- Documented early loss of mitochondrial membrane potential by DMSP in PC-3 cells; further, documented equivalent response in BCL-2 over-expressing PC-3 transfectants
- Documented early DMSP-induced loss of mitochondrial cytochrome C in PC-3 cells, in a time- and dose-dependent manner

REPORTABLE OUTCOMES

An abstract entitled "Dimethyl-sphingosine overcomes multiple mechanisms of apoptotic resistance in tumor cells" by J. Klostergaard, M.E. Leroux, M. Khodadadian, E. Auzenne, R. Evans, N. Hail, Jr., and T. McDonnell is being presented at the 2004 AACR meeting, March 27-31, in Orlando, FL.

CONCLUSIONS

Human prostatic carcinoma cell lines, PC-3 and bone metastases-derived MDA-PCa-2a and MDA-PCa-2b, are susceptible *in vitro* to Taxol, PGA-TXL and to HA-TXL. Paclitaxel-induced activation of apoptosis can be blocked at the level of caspase-3 induction by S-1-P. DMSP effectively inhibits SpK, reducing cellular S-1-P levels, and thereby facilitating maturation of apoptotic signals. We have also shown that the mitochondria are proximal targets of DMSP-mediated apoptosis in PC-3 cells, resulting in rapid loss of membrane potential, cytochrome C mobilization, and caspase activation; these responses are unaffected by BCL-2, of significant relevance to metastatic prostate cancer. We will continue with the planned Tasks, but will also attempt to merge them with strategies to systemically deliver DMSP, and thereby enhance paclitaxel-induced apoptosis and reverse tumor angiogenesis.

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